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Differential Induction of Nuclear factor-like 2 Signature Genes with Toll-Like Receptors Stimulation

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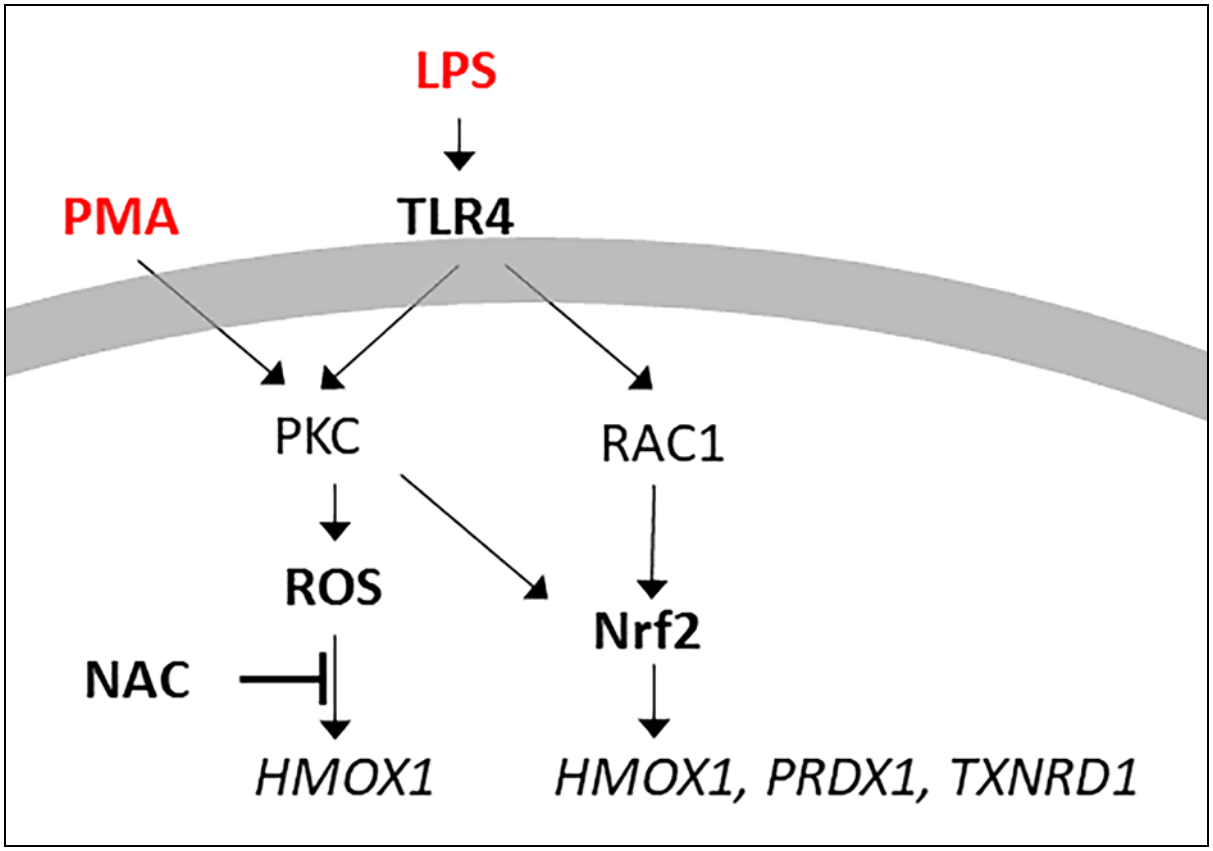
Abstract

Inflammation is associated with production of reactive oxygen species (ROS) and results in the induction of thioredoxin (TXN) and peroxiredoxins (PRDXs) and activation of nuclear factor-like 2 (Nrf2). In this study we have used the mouse RAW 264.7 macrophage and the human THP-1 monocyte cell line to investigate the pattern of expression of three Nrf2 target genes, PRDX1, TXN reductase (TXNRD1) and heme oxygenase (HMOX1), by activation of different Toll-like receptors (TLR). We found that, while the TLR4 agonist lipopolysaccharide (LPS) induces all three genes, the pattern of induction with agonists for TLR1/2, TLR3, TLR2/6 and TLR7/8 differs depending on the gene and the cell line. In all cases, the extent of induction was HMOX1>TXNRD1>PRDX1. Since LPS was a good inducer of all genes in both cell lines, we studied the mechanisms mediating LPS induction of the three genes using mouse RAW 264.7 cells. To assess the role of ROS we used the antioxidant N-acetylcysteine (NAC). Only LPS induction of HMOX1 was inhibited by NAC while that of TXNRD1 and PRDX1 was unaffected. These three genes were also induced by phorbol myristate acetate (PMA), a ROS-inducer acting by activation of protein kinase C (PKC). The protein kinase inhibitor staurosporine inhibited the induction of all three genes by PMA but only that of HMOX1 by LPS. This indicates that activation of these genes by inflammatory agents is regulated by different mechanisms involving either ROS or protein kinases, or both.

Keywords: Nrf2, Toll-like receptors, antioxidant, inflammation, peroxiredoxin, thioredoxin

Graphical abstract



Introduction

Toll-like receptors (TLRs) respond to various pathogen-associated molecular patterns [1, 2] as well as damage-associated molecular patterns released in response to damage or stress [3], leading to the production of proinflammatory cytokines [1, 2]. TLR stimulation increases generation of reactive oxygen species (ROS) which have multiple roles in inflammation, including pathogen defence, redox signalling [4] and activation of redox-sensitive transcription factors including NF-κB and nuclear factor-like 2 (Nrf2; encoded by the gene nuclear factor erythroid-derived 2-Like 2, NFE2L2) [5, 6]. Inflammatory stimuli activate the expression of protein thiol-disulfide oxidoreductases (PDORs) Nrf2 target genes, including thioredoxin (TXN) and peroxiredoxins (PRDXs), along with heme oxygenase (HMOX1) [6].

In addition to their well known antioxidant action [7], PDORs can regulate inflammation. PRDX overexpression inhibits production of inflammatory cytokines in RAW 264.7 mouse macrophages [8], while extracellular PRDX induces the production of inflammatory cytokines [9, 10]. Likewise, TXN overexpression can dampen inflammation [11], but extracellular TXN can act as a chemokine [12] and participate in neutrophil migration.

This aim of this study was to investigate whether activation of different TLRs results in a different pattern of induction of *PRDX1, TXNRD1*, and *HMOX1*, using mouse RAW 264.7 macrophage and human THP-1 monocyte cell lines. We also studied the role of protein kinase C (PKC) and ROS in their induction using phorbol myristate acetate (PMA) that stimulates production of reactive oxygen species (ROS) via protein kinase C (PKC) [13], or the thiol antioxidant N-acetylcysteine (NAC).

Methods

All chemicals and solutions were purchased from Sigma-Aldrich Corporation (St. Louis, USA) unless otherwise stated.

2.1 Cell culture

RAW 264.7 and THP-1 cells were maintained at 37°C and 5% CO_2 in RPMI containing 2mM L-glutamine, supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, USA) and 10% heat inactivated

foetal calf serum. For experiments RAW cells were seeded at $1.25x10^5$/ml and THP-1 cells at $5x10^5$/ml in 24- or 96-well plates. RAW cells were incubated overnight to allow adhesion prior to TLR stimulation; THP-1 cells were stimulated one hour after seeding.

TLR stimulation was carried out for 6h (RT-qPCR) or 24h (tumour necrosis factor (TNF) production) using the following TLR agonists: the synthetic tripalmitoylated lipohexapeptide analogue of the immunologically active N-terminal portion of bacterial lipoprotein Pam3 (TLR1/2; 1 µg/ml in RAW and 100 ng/ml in THP-1; InvivoGen, Toulouse, France), polyinosinic-polycytidylic acid (poly IC) (TLR3; 12.5 µg/ml; InvivoGen), LPS (TLR4; 10 ng/ml in RAW and 100 ng/ml in THP-1; 055:B5), neoseptin (TLR4; 25 µM), FSL-1 (TLR2/6; 200 ng/ml in RAW and 20 ng/ml in THP-1; InvivoGen), R-848 (TLR7/8; 1 µg/ml in RAW and 10 µg/ml in THP-1; InvivoGen), CL075 (TLR7/8; 10 µg/ml; InvivoGen), ODN 2216 (TLR9; 5 µM; InvivoGen). PMA was used at 500 ng/ml. NAC (10 mM) or staurosporine (100 nM) were applied to cells prior to TLR stimulation for 1h and 30mins respectively. TNF production was quantified using a DuoSet ELISA kit (Bio-Techne, Minneapolis, Minnesota, USA).

2.2 TaqMan RT-qPCR

Total RNA was extracted from cells using QIAzol (QIAGEN, Hilden, Germany), following manufacturer instructions. Reverse transcription (RT) and TaqMan qPCR analysis of *PRDX1* (mouse, Mm01621996_s1; human, Hs00602020_mH), *TXNRD1* (mouse, Mm00443675_m1; human, Hs00917067_m1) and *HMOX1* (mouse, Mm00516005_m1; human, Hs01110250_m1) gene expression was performed as previously reported [14]. Gene expression was quantified using the comparative threshold cycle method, according to Applied Biosystems' guideline. Results were normalised to *HPRT1* (mouse, Mm00446968_m1; human, Hs99999909_m1) and expressed as fold change of one of the control samples, chosen as the calibrator. All TaqMan gene expression assays were purchased from Life Technologies (Carlsbad, USA).

Results

3.1 Differential induction of PRDX1, TXNRD1 and HMOX1 by TLR agonists

Expression of *PRDX1*, *TXNRD1* and *HMOX1* expression was investigated in mouse RAW 264.7 cells following 6h stimulation with each TLR agonist. The doses were selected based on previous studies [14-21]. We confirmed that at these doses all TLR ligands induced significant amounts of TNF in RAW 264.7 cells after 24h stimulation (data not shown), as previously reported in other cell types [19, 22-24].

LPS increased *PRDX1* expression 2.5-fold, while other TLR agonists had no effect (Figure 1A). In contrast, *TXNRD1* expression was increased in response to LPS (TLR4), CL075 (TLR7/8) and R-848 (TLR7/8), and *HMOX1* expression was increased by LPS (TLR4), Pam3 (TLR1/2), poly IC (TLR3), CL075 (TLR7/8) and R-848 (TLR7/8), indicating that each of these genes is regulated differently (Figures 1B and 1C).

The pattern of induction of *PRDX1*, *TXNRD1* and *HMOX1* in human THP-1 cells was different, as shown in Figure 2. RAW 264.7 cells are more sensitive to stimulation with LPS than THP-1 cells; therefore, the concentration of LPS was increased 10-fold, from 10 ng/ml in RAW cells to 100 ng/ml in THP-1, in order to achieve significant levels of TNF production (about 700 pg/ml, not shown). On the other hand, the concentrations of Pam3 (TLR1/2) and FSL-1 (TLR2/6) were decreased 10-fold, from 1 μg/ml and 200 ng/ml in RAW cells to 100 ng/ml and 20 ng/ml in THP-1 cells respectively, to elicit production of TNF levels comparable to LPS (not shown).

Unlike RAW 264.7 cells, where only LPS induced *PRDX1*, induction of *PRDX1* was observed also with Pam3 and FSL-1. Induction of *PRDX1* was at most about 2-fold whereas induction of *TXNRD1* was between 3- and 4-fold with LPS, Pam3 or FSL-1. The effect was much stronger on *HMOX1*, with Pam3 and FSL-1 inducing it about 15-fold and LPS 30-fold.

Therefore, like in mouse RAW 264.7 cells, LPS induced the expression of all genes. The main difference between human THP-1 cells and mouse RAW 264.7 cells was that in THP-1 cells Pam3 or FSL-1 were also effective in inducing all three genes; this is not surprising given the high sensitivity of THP-1 cells to stimulation with TLR1/2 and TLR2/6 agonists [25, 26].

Both CLO75 and R-848 induced low levels of *HMOX1* (*HMOX1* mRNA, fold change vs control, mean ± SD, N=4; CLO75: 3.5 ± 0.1; R-848: 3.1 ± 0.3), significantly different from control levels when analysed by Student's t-test (both $P<0.001$ vs control); however, significance was lost after correction for multiple comparisons (Figure 2). Exactly the same picture was obtained in terms of TNF levels (not shown). Of note, R-848 concentration was increased from 1 µg/ml in RAW 264.7 cells to 10 µg/ml in THP-1 to achieve at least some TNF production (about 100 pg/ml, not shown); CLO75 concentration was maintained at 10 µg/ml as in RAW cells since already high, after verifying that it induced some TNF production also in THP-1 cells (about 30 pg/ml, not shown). Low expression of TLR7 and TLR8 in THP-1 cells, and low sensitivity to stimulation with TLR7/8 agonists, have been previously reported [25, 27].

In addition, poly IC (TLR3) which induced *HMOX1* expression in RAW 264.7 cells (Figure 1), did not have any effect in THP-1 cells (Figure 2), maybe due to the low TLR3 expression in THP-1 cells [27]. As in RAW 264.7 cells, stimulation with the TLR9 agonist ODN2216 was ineffective (Figures 1 and 2).

Since LPS was a good inducer of all genes both in human THP-1 cells and in mouse RAW 264.7 cells, we investigated the mechanisms mediating LPS induction of the three genes using the RAW 264.7 cells as a model.

To confirm whether LPS induction was mediated by TLR4, we used neoseptin, a synthetic TLR4 agonist [17]. As shown in Figure 3, neoseptin induced expression of each of the investigated genes in RAW 264.7 cells to an equal or greater extent than LPS.

3.2 Involvement of ROS and protein kinases

To investigate the role of ROS we used an antioxidant (NAC) or a TLR-independent inducer of ROS (PMA) [28]. PMA induced all three genes, to a similar extent as LPS (Figure 4 A-C). It should be noted that, with both stimulants, the extent of induction of *HMOX1* was greater, in all experiments, than that of *PRDX1* or *TXNRD1*. *PRDX1* and *TXNRD1* gene expression was unaffected by NAC treatment (Figure 4A, B) suggesting that, in our experimental model, the increased expression of these genes

is not mediated via LPS- or PMA-induced ROS. However, *HMOX1* gene expression decreased with NAC treatment, suggesting that ROS are involved in the regulation of *HMOX1* by LPS and PMA (Figure 4C).

Because both PMA and LPS activate PKC, and this mediates ROS production in activated RAW264.7 cells [29], we tested the effect of staurosporine, a pan-specific protein kinase inhibitor. Staurosporine, added for 30mins prior to the addition of PMA, decreased induction of all genes (Figure 4D-F), suggesting that PKC is involved in PMA-induced gene expression. However, it decreased LPS-induced *HMOX1* expression (Figure 4F) but not *PRDX1* or *TXNRD1* (Figure 4D, E), indicating the existence of differential mechanisms for LPS induction of the three genes.

Discussion

Our results indicate that, although *PRDX1*, *TXNRD1*, and *HMOX1* are all Nrf2 target genes, each of them had unique expression profiles in response to activation of different TLRs. The pattern of induction of *PRDX1*, *TXNRD1* and *HMOX1* by TLRs differs between THP-1 and RAW 264.7 cells. While induction of all three genes by TLR4 is validated across both models, Pam3 (TLR1/2) and FSL-1 (TLR2/6) induce all genes in THP-1 cells whereas in RAW 264.7 cells only induction of *HMOX1* by Pam3 is observed. In addition, CL075 and R-848 (both TLR7/8) are effective only in the mouse system; this is probably reflecting a more general lower responsiveness of THP-1 cells to TLR7/8 stimulation [25]. In both the human and mouse cell lines tested, *HMOX1* was the gene which was induced the most, the extent of induction being *HMOX1*>*TXNRD1*>*PRDX1*.

Nrf2-mediated induction of *HMOX1* has been previously reported in RAW 264.7 cells following stimulation of TLR2/6, TLR4, TLR7 and TLR9 [30]. It should be noted, however, that although *HMOX1* is considered a prototypic Nrf2 target gene, it can also be induced by LPS via Nrf2-independent pathways as a partial induction of *HMOX1* by LPS is also observed in the presence of Nrf2 siRNA [31]. Others have shown that, *in vivo,* LPS is a more potent inducer, in terms of fold induction in the liver, of *HMOX1* than *PRDX1*, while both genes are induced to the same extent by the electrophile butylated hydroxyanisole [32], suggesting an additional mechanism to that mediated by Nrf2. Altogether, these observations all point to a higher

inducibility of *HMOX1*, and could help explain the higher level of induction we observed with *HMOX1* compared to *PRDX1* and *TXNRD1*.

Additionally, although Nrf2 is a transcription factor common to all three target genes, and we often view them only as Nrf2 targets, other transcription factor binding sites can be predicted for each gene. We analyzed the predicted regulatory motifs in the three mouse genes using oPOSSUM version 3.0 [33]; several transcription factors binding sites were identified, of which Nrf2 was the only one in common to all three genes, as shown in Table 1.

Table 1. Transcription factor binding sites for *HMOX1*, *PRDX1* and *TXNRD1*

Gene	Transcription factors
HMOX1	Sox2, HNF1A, Pou5f1, MYC::MAX, HNF1B, NHLH1, NFE2L2, Arnt, Nr2e3, TAL1::TCF3, MAX, Mycn, Myc, USF1
PRDX1	NFE2L2, PPARG::RXRA, HNF4A, TBP, EBF1, SP1, SOX9, FOXI1, CEBPA, Klf4, MZF1_5-13, Hand1::Tcfe2a, Sox5, NKX3-1, Nobox, Gfi, Arnt::Ahr, FOXA1, RUNX1, NR4A2, Sox17
TXNRD1	Sox2, Pou5f1, TEAD1, HLF, Egr1, NFE2L2, IRF1, STAT1, MEF2A, NFIL3, Nr2e3, RELA, INSM1, TAL1::TCF3, NF-kappaB, MAX, Foxq1, Zfx, RORA_1, CREB1

Transcription factor binding sites with a Fisher score > mean+1*SD, predicted using oPOSSUM version 3.0 [33]. Nrf2 is termed NFE2L2 in the table.

This, along with our findings on the differential regulations of these genes by different TLRs, indicates that considering *PRDX1*, *HMOX1* and *TXNRD1* purely as Nrf2 targets is an oversimplification. Further work will be required to identify the differential role of the transcription factors involved in the induction of these genes by LPS and other inflammatory agents.

Our finding that the expression of the three genes can be induced with PMA suggests a role for PKC, as confirmed by inhibition of PMA-induced expression of all genes by staurosporine. This does not exclude an involvement of Nrf2, which can be activated through PKC-induced phosphorylation [34]; of note, staurosporine inhibits

PMA-induced transcription of Nrf2-target genes [35]. PKC can be activated by LPS [36], but staurosporine inhibits only LPS-induced *HMOX1* and not *PRDX1* and *TXNRD1*; this suggests that PKC might mediate only LPS induction of *HMOX1*, affecting a pathway partly independent of Nrf2, since it only induces *HMOX1*. However, it should be mentioned that, although staurosporine is widely used as a PKC inhibitor, it can also inhibit other protein kinases [37].

The potential role of ROS is another issue that needs to be clarified. Previous studies have shown that LPS can induce Nrf2 activation independently of ROS [31], possibly through the GTP-binding protein RAC1 [38]. Our experiments using the antioxidant NAC showed that NAC had no effect on LPS- or PMA-induced *PRDX1* nor on *TXNRD1* expression, but did reduce *HMOX1* expression suggesting that this is a more redox-sensitive gene (Figure 4). It is known that *PRDX1*, *TXNRD1* and *HMOX1* are induced by oxidative stress, but the results presented here suggest that some genes may be more susceptible to changes in redox state than others [39]. A study with HOCl has shown that *HMOX1* is induced at lower HOCl concentrations, with the extent of induction similar to the one we observed: *HMOX1>TXNRD1>PRDX1* (Supplementary file 2 in [40]).

Elucidating the regulation of PDORs in inflammation may be important in exploring new avenues for the therapy of inflammatory disease. In this respect, it is important to remember that an inhibitor of TXNRD, auranofin, is used in the therapy of rheumatoid arthritis [41] while the Nrf2 activator dimethyl fumarate is used in the treatment of multiple sclerosis [42, 43]. The identification of pathways that fine-tune TLR responses may be important in the development of anti-inflammatory agents that have a lesser impact on the innate immunity against pathogens.

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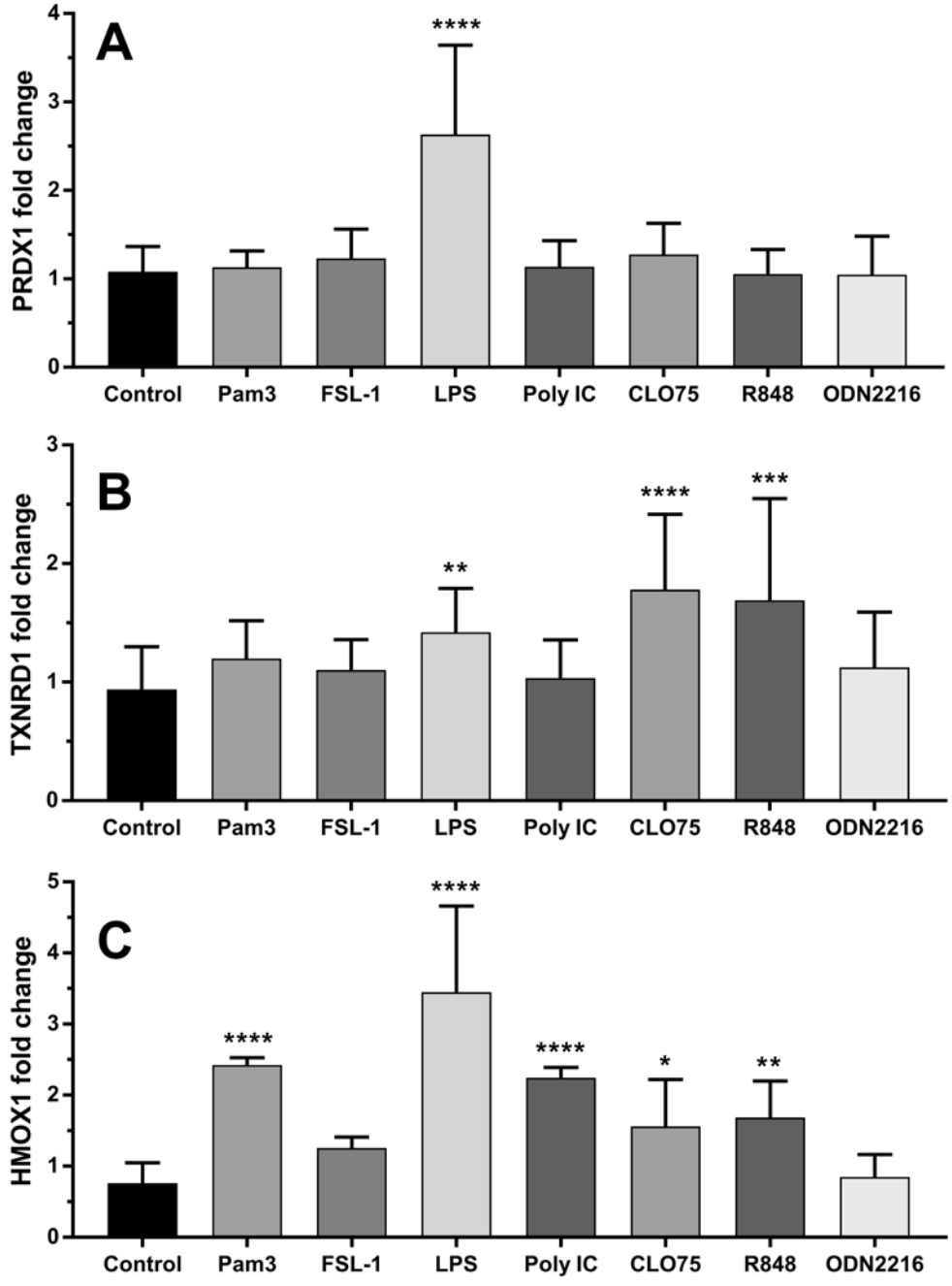

Figure 1. Nrf2 target gene expression induced by agonists of different TLRs in mouse RAW 264.7 cells. TaqMan qPCR analysis of *PRDX1* (A), *TXNRD1* (B) and *HMOX1* (C) gene expression in RAW 264.7 cells stimulated with TLR agonists as described in Methods. Data were normalised to *HPRT1* and expressed as fold change vs one control sample ($n \geq 8$, mean±SD). ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 vs control by one-way ANOVA followed by Dunnett's multiple comparisons test.

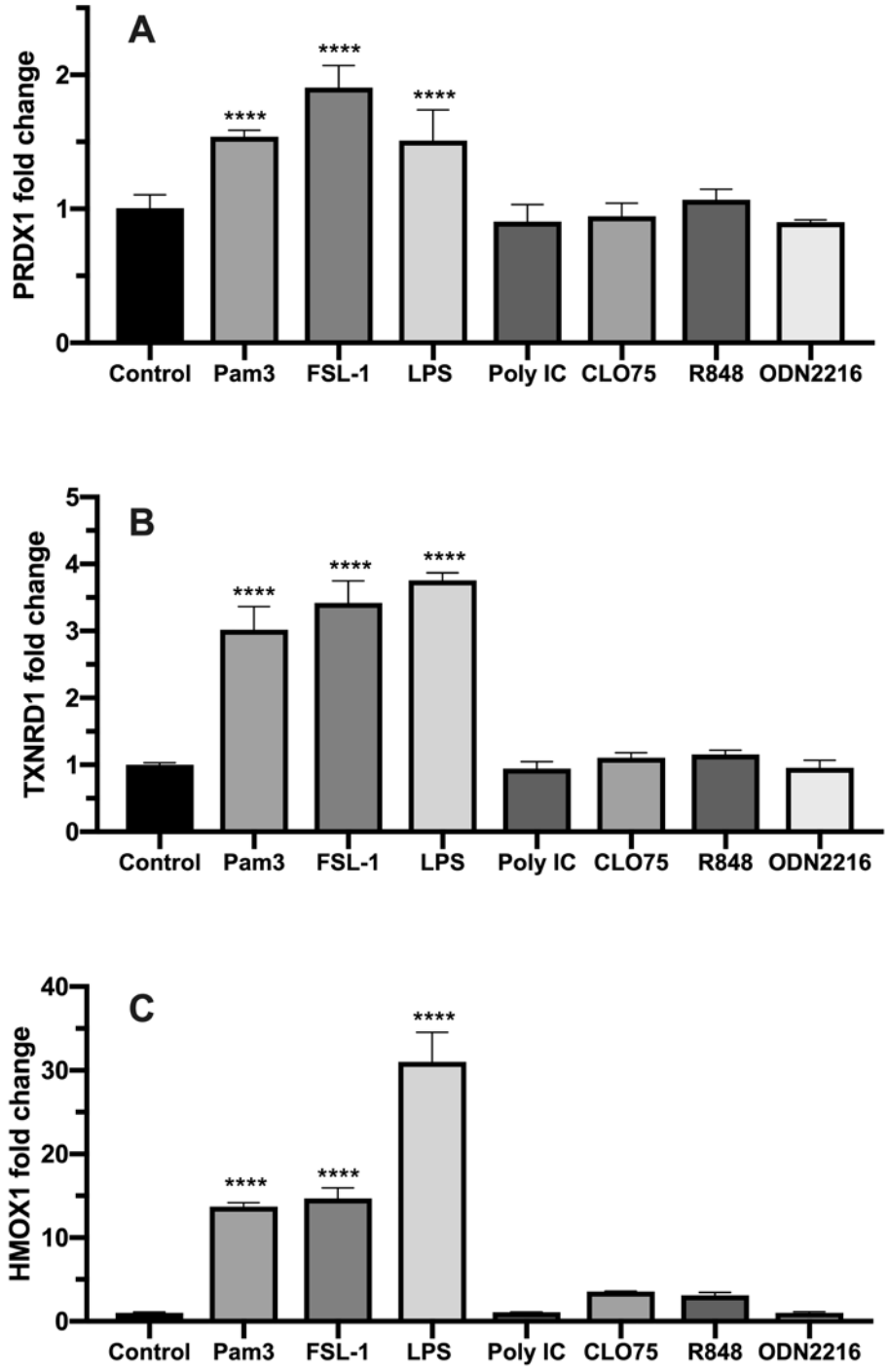

Figure 2. Nrf2 target gene expression induced by agonists of different TLRs in human THP-1 cells. TaqMan qPCR analysis of *PRDX1* (A), *TXNRD1* (B) and *HMOX1* (C) gene expression in THP-1 cells stimulated with TLR agonists as described in Methods. Data were normalized to *HPRT1* and expressed as fold change vs one control sample (n=4, mean±SD). ****P<0.0001 vs control by one-way ANOVA followed by Dunnett's multiple comparisons test.

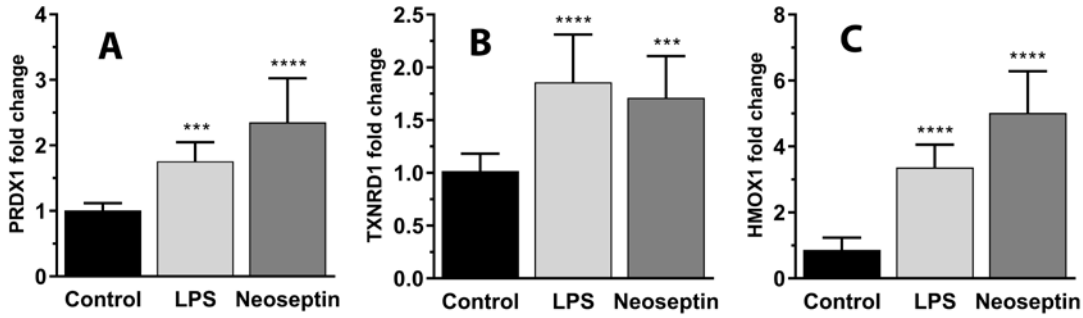

Figure 3. *PRDX1*, *TXNRD1* and *HMOX1* gene expression is increased with the TLR4 agonist neoseptin. TaqMan qPCR analysis of *PRDX1* (A), *TXNRD1* (B) and *HMOX1* (C) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml) or neoseptin (25μM). Gene expression values were normalised to *HPRT1* and expressed as fold change vs one control sample (n=12, mean±SD). ****P<0.0001, ***P<0.001 vs control by one-way ANOVA followed by Dunnett's multiple comparisons test.

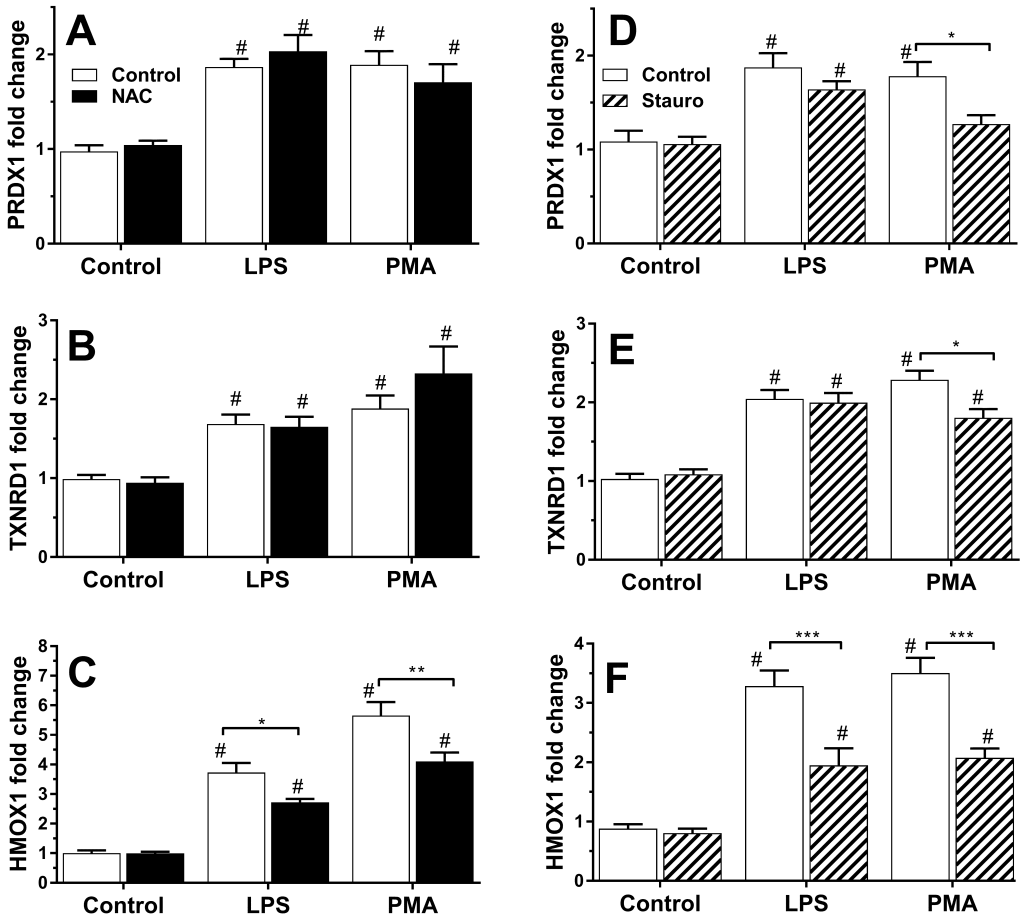

Figure 4. Effect of NAC and the PKC inhibitor staurosporine on LPS- and PMA-induced *PRDX1*, *TXNRD1* and *HMOX1* gene expression. TaqMan qPCR analysis of *PRDX1* (A,D), *TXNRD1* (B,E) and *HMOX1* (C,F) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml) or PMA (500ng/ml) with or without 10mM NAC (A,B,C) or 100 mM staurosporine (D,E,F). White bars, control; black, NAC; hatched, staurosporine. Gene expression values were normalised to *HPRT1* and expressed as fold change vs one control sample (*n*=12, mean±SD). #P<0.05 vs untreated cells; ***P<0.001, *P<0.05 vs control by two-way ANOVA and Tukey's test for multiple comparisons.